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SHORT COMMUNICATION

Synthetic circuit of inositol phosphorylceramide synthase in *Leishmania*: a chemical biology approach

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Abstract Building circuits and studying their behavior in cells is a major goal of systems and synthetic biology. Synthetic biology enables the precise control of cellular states for systems studies, the discovery of novel parts, control strategies, and interactions for the design of robust synthetic systems. To the best of our knowledge, there are no literature reports for the synthetic circuit construction for protozoan parasites. This paper describes the construction of genetic circuit for the targeted enzyme inositol phosphorylceramide synthase belonging to the protozoan parasite *Leishmania*. To explore the dynamic nature of the circuit designed, simulation was done followed by circuit validation by qualitative and quantitative approaches. The genetic circuit designed for inositol phosphorylceramide synthase (Biomodels Database—MODEL1208030000) shows responsiveness, oscillatory and bistable behavior, together with intrinsic robustness.

Keywords IPCS · *Leishmania* · Genetic circuit · Simulation · Degradation rate · Logic circuit

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Introduction

In recent years, the disciplines of systems, synthetic, and chemical biology along with bioinformatics have gained importance as the embodiments of the future of biological sciences. Some of the greatest advances in biology and biotechnology are now arising at the intersection of the top-down systems approach and the bottom-up synthetic approach [1, 2]. Recent systems biology paradigms such as computational systems analysis, methods for quantifying time-dependent gene expression, and bioinformatics cataloging of cellular parts enable synthetic biology. Interdependence of systems and synthetic biology lies in the expansion and automation of the process of parts identification [3, 4]. A major goal of systems biology is to combine these parts to design biological circuits and study their behavior inside the cellular environment. The rapid capacity to sequence new genomes has sparked interest in equally rapid annotation capacities. As a result, databases of prokaryotic [5] and eukaryotic [6] motifs have been curated, enabling the annotation of different promoters [7], transcription factor binding sites, and terminators [8]. In addition to providing a repository of natural biological parts, these tools allow synthetic biologists to design novel regulatory elements by combining motifs in many interesting ways [9].

Biological circuits are built by the assembly of biological parts [10]. A part can be described as the sequence of DNA that encodes certain information, associated with a specific function. Assembly of these parts (promoters, ORFs, ribosome binding sites, etc.) give rise to a device, several such devices make up a system. With a myriad number of biomolecules involved together with their dynamic behavior, there tends be a certain complexity involved within every system. Because of its parts-to-whole approach, a synthetic device is also comparable to an electronic circuit wherein

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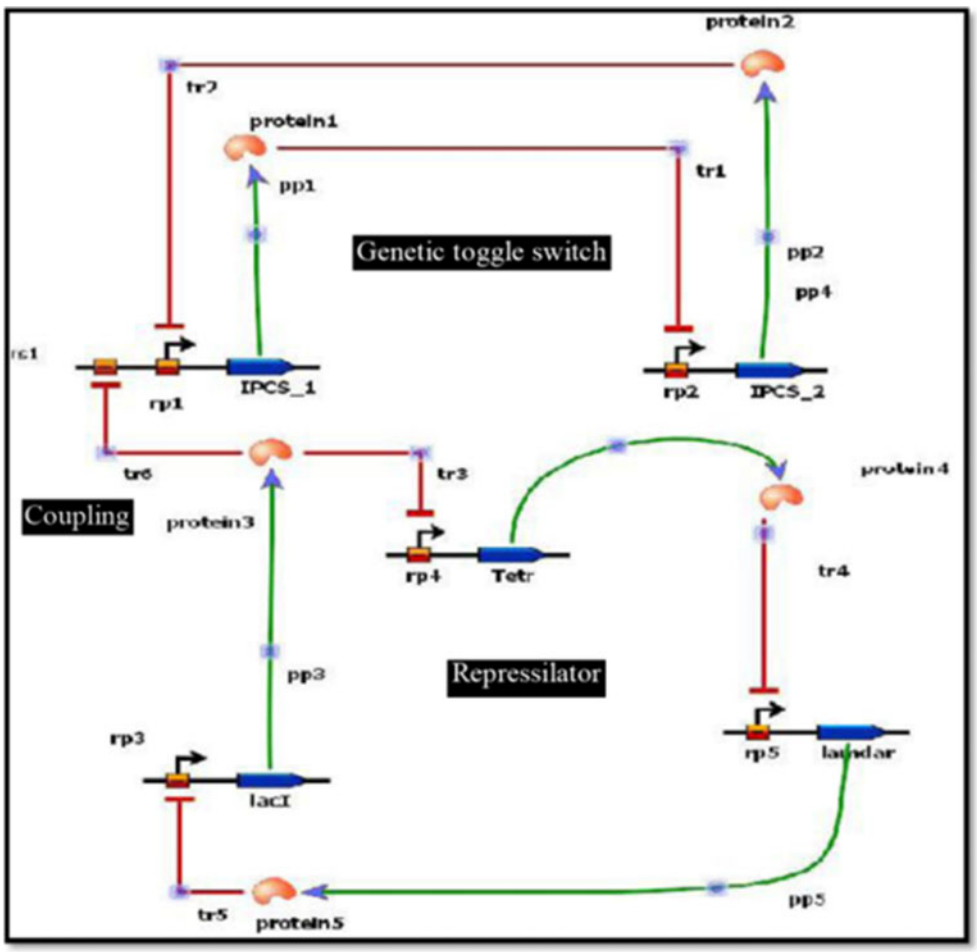
stimuli function as input and the final output is decided by the signals that modulate the behavior of the system [11].

Synthetic biology offers the potential to build constructs like engineered biomolecules, gene networks, and programmable organisms to alter the mechanisms underlying the disease. The first genetic toggle switch was constructed in *Escherichia coli* by Gardner *et al* to understand the bistable and oscillatory behavior of the circuit [1]. Since then, synthetic biology has evolved right from building up of simple toggle switches to complex genetic circuits, replacing or rewiring them to achieve the desired cellular behavior.

One of the many applications of synthetic biology is in the field of infectious diseases. This paper highlights the use of mathematical modeling with deterministic and stochastic approaches as a central component for the synthetic circuit design of inositol phosphorylceramide synthase (IPCS), an enzyme belonging to the sphingolipid metabolism of *Leishmania*. IPC is present together with other sphingolipids and sterols in the membrane microdomains [12].As IPCS is parasite specific and has no functional equivalent in mammals, it can serve as putative drug target for *Leishmaniasis* [13].

Mathematical modeling serves as a critical link between the concept and realization of a biological circuit [14]. The constructed IPCS genetic circuit was modeled and simulated using diverse set of algorithms. This assumption was shown to greatly simplify the stochastic model thereby reducing the computational complexity. Once the circuit was constructed, validation was performed using the qualitative and quantitative approaches. The qualitative behavior of the circuit can be very informative in models with multiple steady states and showing switch-like or oscillatory behavior. It can be studied either over small parts of the parameter space by simply scanning over defined ranges of parameters and the initial conditions or by the global bifurcation analysis. On the other hand, qualitative analysis gives an idea as to which parameters offer the best success in achieving a desired behavior, whether a certain design can exhibit the desired function. Identifying the most crucial parameters depends not only on the mathematical analysis but also on the biological feasibility. Characteristics such as strength promoter, transcript, and protein stability are quite variable. The changes in the characteristics of biological components can at best be



Genetic circuit for IPCS

Fig. 1 Genetic circuit for IPCS includes two genes (IPCS1 and 2) forming the toggle switch, coupled to a repressilator comprising of three genes (LacI, TetR, and LambdaR) [Biomodels Database (MODEL1208030000)]

qualitative; however, it is also important to find parameter ranges that show robustness to variations. Model validity can be checked by comparison of the simulation runs with the quantitative experimental data such as the time course data or by the steady-state analysis [15, 16]. Based on the current approaches dealt, herein, we describe how different approaches of bioinformatics and systems biology could enable novel synthetic biology applications for the treatment of *Leishmaniasis*.

Materials and methods

Circuit design

The genetic circuit was built using Tinker Cell, a Computer-Aided Design Software for synthetic biology (http://www.tinkercell.com). For the construction of the switch for IPCS, homologous sequences of *Leishmania major* were retrieved from GeneDB and Uniprot. Input sequences were in FASTA format or Genbank format. Parts were obtained from the Registry of Biological parts (http://partsregistry.org/cgi/partsdb/Statistics.cgi), which is a collection of genetic parts that can be mixed and matched to build synthetic biology devices and systems. Grouping of the orthologous proteins was done by OrthoMCL. Selected parts were inserted for the construction of a model along with their DNA sequences. The circuit was built using two mutually repressible genes coding for IPCS that constitute the genetic toggle switch and a three repressible promoters that made up the repressilator. Transcription repression reactions were assigned for the two genes that are a part of the toggle switch as they mutually repress each other. Similarly, transcription repression reaction was assigned to each of the three genes that make up the repressilator [17]. Coupling between toggle switch and the repressilator was done by the insertion of the transcription repression reaction between the first gene of the toggle switch and first gene of the repressilator such that gene of the repressilator controls the gene of the toggle switch [18].

Parameters like protein degradation, promoter strength, transcription rate, dissociation constants, and the Hill's coefficient along with the reaction rates for the reaction were set. Parameter scan was done using the steady-state analysis and the genetic circuit was subjected to tau-leap stochastic simulation to study the behavior of the constructed circuit [19]. The logic gates were deciphered once the bistable and the oscillatory behavior of the genetic circuit was achieved. Truth tables obtained were converted into the circuit scheme via the

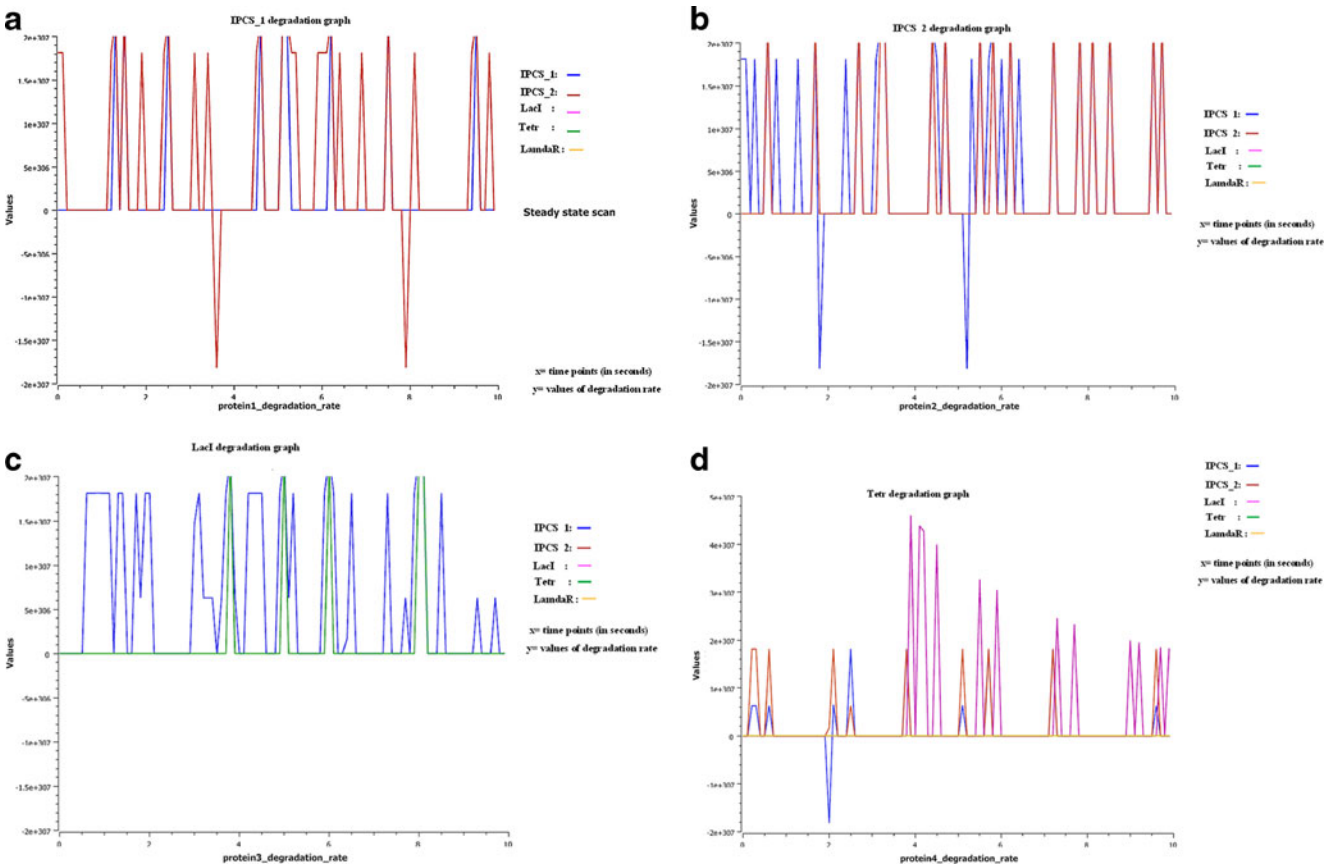


Fig. 2 Degradation graphs for all the proteins after the steady scan analysis. **a** Degradation graph for IPCS 1, **b** degradation graph for IPCS 2, **c** degradation graph for LacI, **d** degradation graph for Tet R

Case 1:Change in the Kd values of IPCS1(Kd =1 and 1.01)

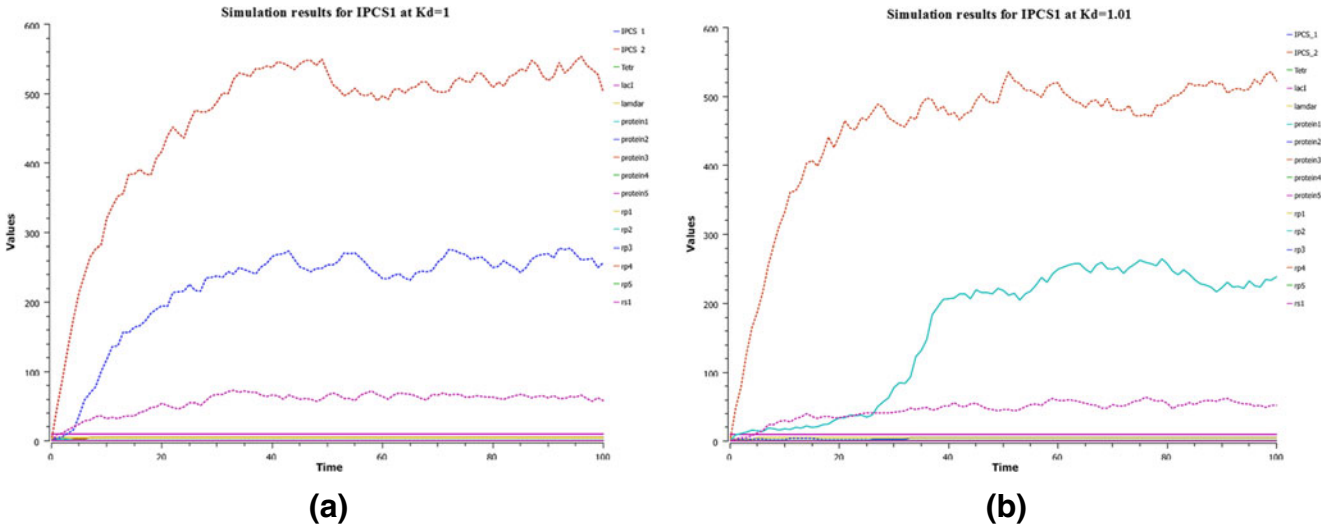


Fig. 3 Graphs depicting the results for the stochastic simulation for the circuit by changing the Kd values of IPCS1

Karnaugh map (K-map), a method to simplify the Boolean algebra expressions (www.karnaugh.html).

Model validation

Bioconductor package based on the R programming language was used for the model validation which includes two packages—(a) Gene Regulatory Network Inference Using Time Series (GRENITS) and (b) Boolnet. The time series data required for the validation was obtained from Complex Pathway Simulator (COPASI; www.copasi.org) by using time course method for the stochastic simulation [20]. Qualitative network modeling was done by the Boolean method using BoolNet. BoolNet helps integrate methods for the synchronous, asynchronous and probabilistic Boolean networks. Apart from the reconstruction of networks from time series data, robustness analysis via perturbation and Markov chain simulations followed by the identification and visualization of attractors can be done [21]. Quantitative network modeling was done by the Bayesian approach using the GRENITS package which uses the time series data generated by COPASI. Network inference using ODE time series data was done which gives the probability of the genes included in the circuit, suggesting the regulators of the

Case 2: Change in the Kd values of IPCS2 (Kd =1.01 and 1.02)

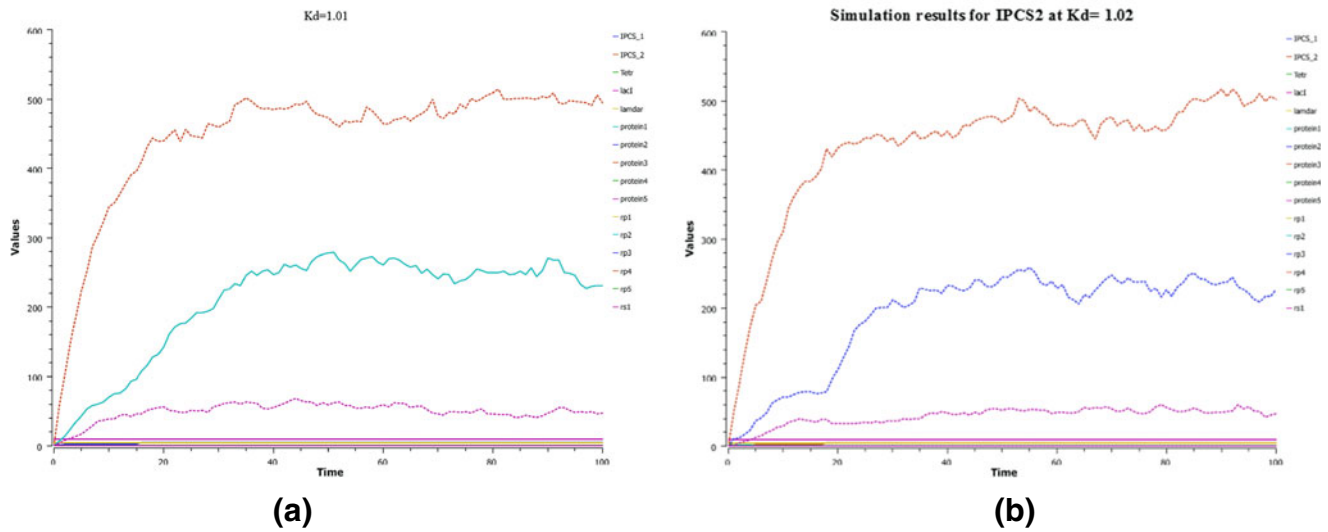


Fig. 4 Graphs depicting the results for the stochastic simulation for the circuit by changing the Kd values of IPCS2

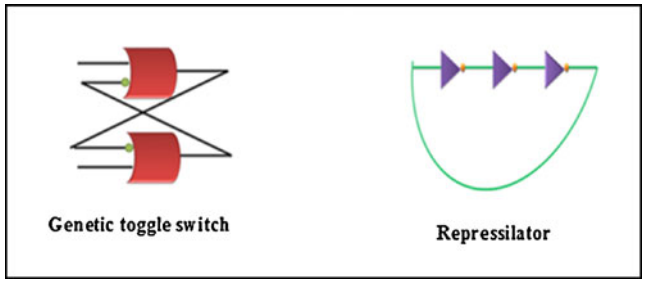


Fig. 5 Logic gates representing the toggle switch (having two IMPLIES gates) and the repressilator (with three NOT gates)

circuit along with the network uncertainty [22]. Pajek, a computer program for the analysis of large networks and visualization of the graphs was used to visualize the transition states network [23].

Results and discussion

Circuit design

The genetic circuit for IPCS is composed of a toggle switch controlled by a repressilator. Genetic toggle switch is made up of two genes (IPCS1 and IPCS2) which code for repressor proteins and mutually repress each other, reflecting the bistable behavior of the toggle switch. The repressilator is made up of three genes namely lactose repressor (LacI), tetracycline repressor (TetR), and the lambda repressor (Phage lambda). The repressilator genes acts in cyclic manner with LacI repressing TetR, TetR repressing

LamdaR and LamdaR in turn represses LacI, producing an oscillatory behavior. Coupling between genetic toggle switch and repressilator was developed between LacI and IPCS1 where LacI regulates the IPCS1 and represses it (Fig. 1).

Steady-state analysis

For the steady-state analysis, default parameters were initially assigned to the genetic modules on the basis of their connectivity in the circuit and the Kd values for the genes were set to 1 (Electronic supplementary material (ESM) Table 1). The translation rates and transcription rates are set to 1 for all the genetic modules. Since every reaction is a transcriptional repression reaction, hill-hinze kinetics were applied to the genetic circuit and the value of hill's co-efficient was set to 2; the kinetics have been included in the ESM. Steady-state scan analysis for the degradation rates of all the proteins was carried out.

The degradation of IPCS1 protein resulted in a rise in the expression of the opposite toggle gene IPCS2. At two time points, there was a peak fall in the level of IPCS2 after its degradation and here a switching behavior was seen (Fig. 2a). This desired graph was obtained at 0.18 degradation rate of IPCS1. Similarly on increasing the levels of IPCS2 at a particular interval of time, there was a decrease in the level of IPCS1 as it was degraded, at these two time points, switching behavior was observed (Fig. 2b). The desired behavior was obtained at 0.08 degradation rate of IPCS2. These two observations indicate the bistable behavior of the circuit wherein it was seen that IPCS1 is capable of repressing IPCS2 and IPCS2 inturn could repress IPCS1 and the flipping of the switch occurs. Initially, there was no coupling between Lac I and IPCS1 I, however at a time interval of 4 sec, there was an increase in the level of LacI along with a drop in the levels of IPCS1. This change in the regulation of IPCS1 with respect to LacI shows the coupling between genetic toggle switch and the repressilator (Fig. 2c). In the degradation graph for TetR, there was a less oscillation between LambdaR and TetR; however, repression of LamdaR was observed (Fig. 2d).

Simulation

Simulation of the genetic circuit was performed for 100 time points using tau-leap stochastic simulation at differernt concentrations. Based on the Kd values, the change in the protein level were studied, the results of fluctuations in the protein level with respect to change in dissociation constant (Kd) are dealt in two cases (cases 1 and 2). In case1, it was observed that when the Kd value was 1, the level of IPCS1 was increased but when the value of Kd rose to 1.01,there was a switch in the level of IPCS2 as the dissociation between IPCS1 and regulatory binding site increased which lead the circuit to toggle increasing the levels of IPCS2. Similar fluctuations in protein level were also observed when the Kd values at regular intervals are altered (Fig. 3, ESM Fig. 11.2). Similarly in case 2, when Kd value was set

Table 1 Truth table for the genetic circuit, A and B are the input signals of the toggle switch and C represents the input signal of the repressilator

Input (A)	Input (B)	Input (C)	Output
0	0	0	1
0	0	1	0
0	1	0	1
0	1	1	0
1	0	0	0
1	0	1	0
1	1	0	0
1	1	1	0

Table 2 Karnaugh map of the truth table of the genetic circuit

C	AB				
	00	01	11	10	
	0	1	1	1	0
	1	0	0	0	0

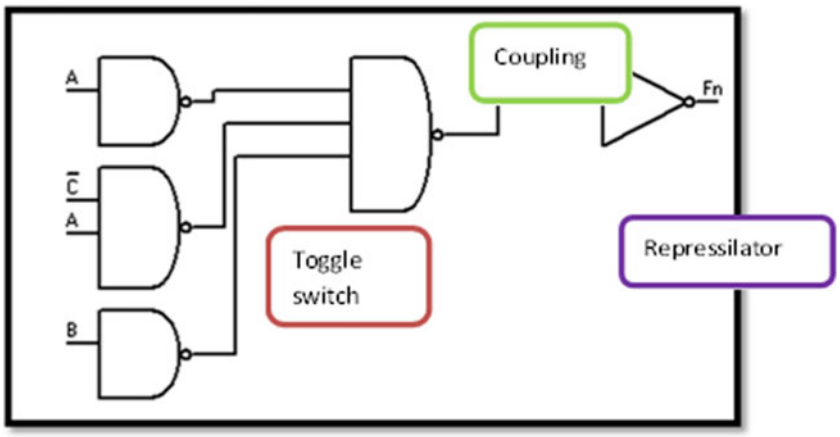


Fig. 6 Digital circuit showing the coupling behavior of the toggle switch and the repressilator

to 1.01, the level of IPCS2 was high but when the value of Kd rose to 1.02, a switch in the level of IPCS1 was observed as the dissociation between IPCS2 and repressor increased. This caused the circuit to flip and level of IPCS1 increased. Similar perturbations in the levels of IPCS1 and IPCS2 were also observed when there was a change in the Kd at regular intervals (Fig. 4, ESM Fig. 11.3). These observations indicate that there is flipping of the toggle switch indicating that the constructed toggle switch comprising of IPCS1 and 2 exhibits bistable behavior and that there was a direct coupling between IPCS1 of the toggle switch and LacI of the repressilator.

Digital circuit representation of the synthetic genetic circuit

Digital circuits gives valuable insight into the logic gates implemented in the circuit design to understand the functioning of the circuit. In comparison to an electronic switch, the two mutually repressible genes constituting the toggle switch are comparable to two IMPLIES gates such that output of one IMPLIES gate is the input signal for the opposite IMPLIES gate. On the other hand, the repressilator is constructed by integrating an odd number of NOT gates in a circular fashion such that the output of the one is the input of another and the output of the last gate serves as an input of the first one (Fig. 5). Odd number of NOT gates are included in the repressilator such that the last output signal oscillates between high and low alternatively.

The logic behind the IPCS genetic circuit can be represented by a truth table (Table 1). Karnaugh map for the circuit was generated which is a simplified representation of the truth table that allows the conversion of the truth table into a Boolean formula (Table 2). This method derives two different descriptions of every digital circuit that are called as product of sums (POS) and sum of products (SOP). Digital circuit shows the logic gate for the toggle switch and repressilator with the SOP=($|A \times C$)+($|A \times |B$) and POS=$|A \times (|B+C)$. The circuit scheme for the circuit is represented in Fig. 6.

Genetic circuit model validation

Model validation was done by using qualitative and quantitative approaches. ODE model for the genetic circuit was generated giving an insight into regulatory mechanism for the circuit. The equations for the construction of ODE model are included in the ESM.

Network inference

Gene regulatory network was constructed for the genetic circuit using the Bioconductor package. The model was simulated using COPASI to obtain the time series data for qualitative and quantitative network modeling. Linear network construction was done as the circuit model was found to be stable. Qualitative network modeling was done using the GRENITS package which gave the probability of each regulator in the regulatory network circuit. Monte Carlo–Markov chain simulation was performed using the default parameters to obtain the posterior probability (Fig. 8). During the simulation, two Markov chains are generated and based on their convergence the link probability of the network is deduced. A probability matrix for each gene in the

Table 3 Posterior probabilities for each network connection

From	To	Probability
IPCS_1	IPCS_1	0
IPCS_1	IPCS_2	1
IPCS_1	Tetr	0
IPCS_1	lacI	0
IPCS_1	LamdaR	0
lacI	IPCS_1	1
lacI	Tetr	0
lacI	lamdaR	1
LamdaR	IPCS_1	1
LamdaR	IPCS_2	0

Table 4 Posterior probability for number of regulator for each gene, 1 represents regulation between the regulators and 0 indicates no regulation

	1 Regulators	2 Regulators	3 Regulators	4 Regulators	5 Regulators
IPCS_1	0	1	1	0	0
IPCS_1	1	0	0	0	0
Tetr	1	0	0	0	0
lacI	1	0	0	0	0
LamdaR	0	1	0	0	0

regulatory network, an analysis and a convergence plot was generated for the network circuit. Network inference was made for 10 and 100 time points, the network inference was observed to understand the regulatory mechanism in the circuit wherein a probability of 1 shows the regulation between the respective regulators, while a probability of 0 indicates no regulation. A probability of 1 was obtained between IPCS1 and 2 and they appear to be regulating each other; in addition, a probability of 1 was also seen between LacI and IPCS 1 reflecting the coupling mechanism (Tables 3 and 4).

Analysis plot of the genetic circuit shows the link probability which provides insight into the switching behavior, i.e., between the LacI belonging to the repressilator and IPCS1 of genetic toggle switch. A probability of 1 is represented by blue color in the plot. The analysis plot shows the

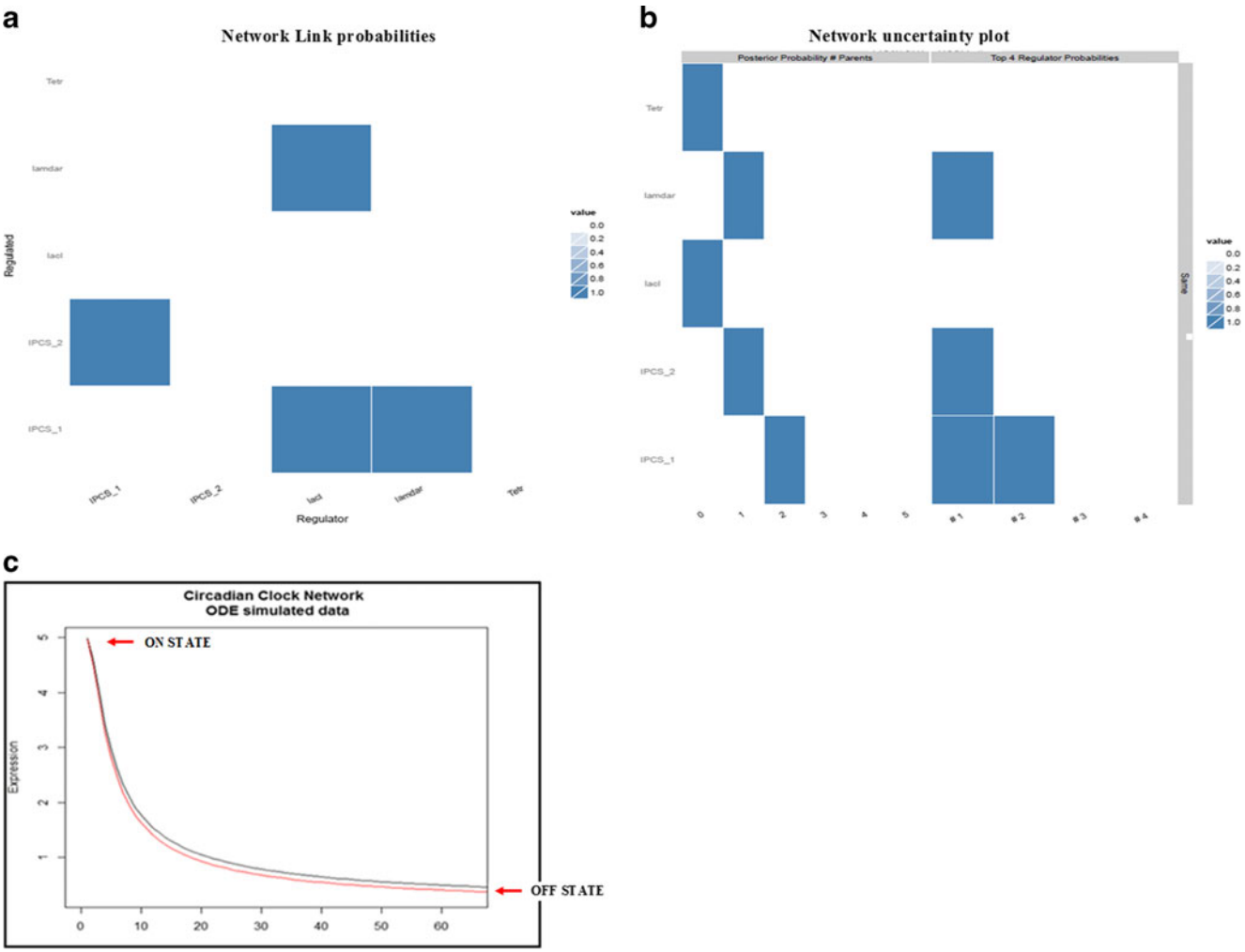


Fig. 7 **a** Heat map plot of Network Link probabilities, **b** network uncertainty plot, **c** circadian clock network showing the switching behavior of IPCS1 and IPCS2

probability of 1 between the two genes IPCS1 and IPCS2. It was also observed that the LacI and IPCS1 genes appear to be linked as the probability between them was also 1 (Fig. 7a). Network uncertainty is given by the marginal network uncertainty plot giving and idea about the top regulators in the circuit. From the network uncertainty plot, IPCS1, IPCS 2 together with LacI were the key regulators in the network model (Fig. 7b, c).

Convergence plots The posterior means of the variables (gamma, B, lambda, and mu) each are compared in Fig. 8.

Inferred network The inferred network displays the regulation between IPCS1 and IPCS2 which reflects the switching behavior of the toggle switch. Network inference made at 10s time point shows no regulation between LacI and IPCS1 and there was no coupling seen between the toggle switch and repressilator. It was observed that IPCS2 repressed the gene expression of IPCS1 while LacI and TetR regulated the expression of LambdaR. IPCS1 remained in an ON state while IPCS2 was in an OFF state (Fig. 9a). However, at the second time point, i.e., network inference made at 100s, coupling between the toggle switch and the repressilator was seen which resulted into a repression of IPCS1 by LacI, with IPCS1 in OFF state and IPCS2 in ON state (Fig. 9b). Thus, the probability derived justifies the associated parameters that accounts for the designability of the IPCS genetic circuit.

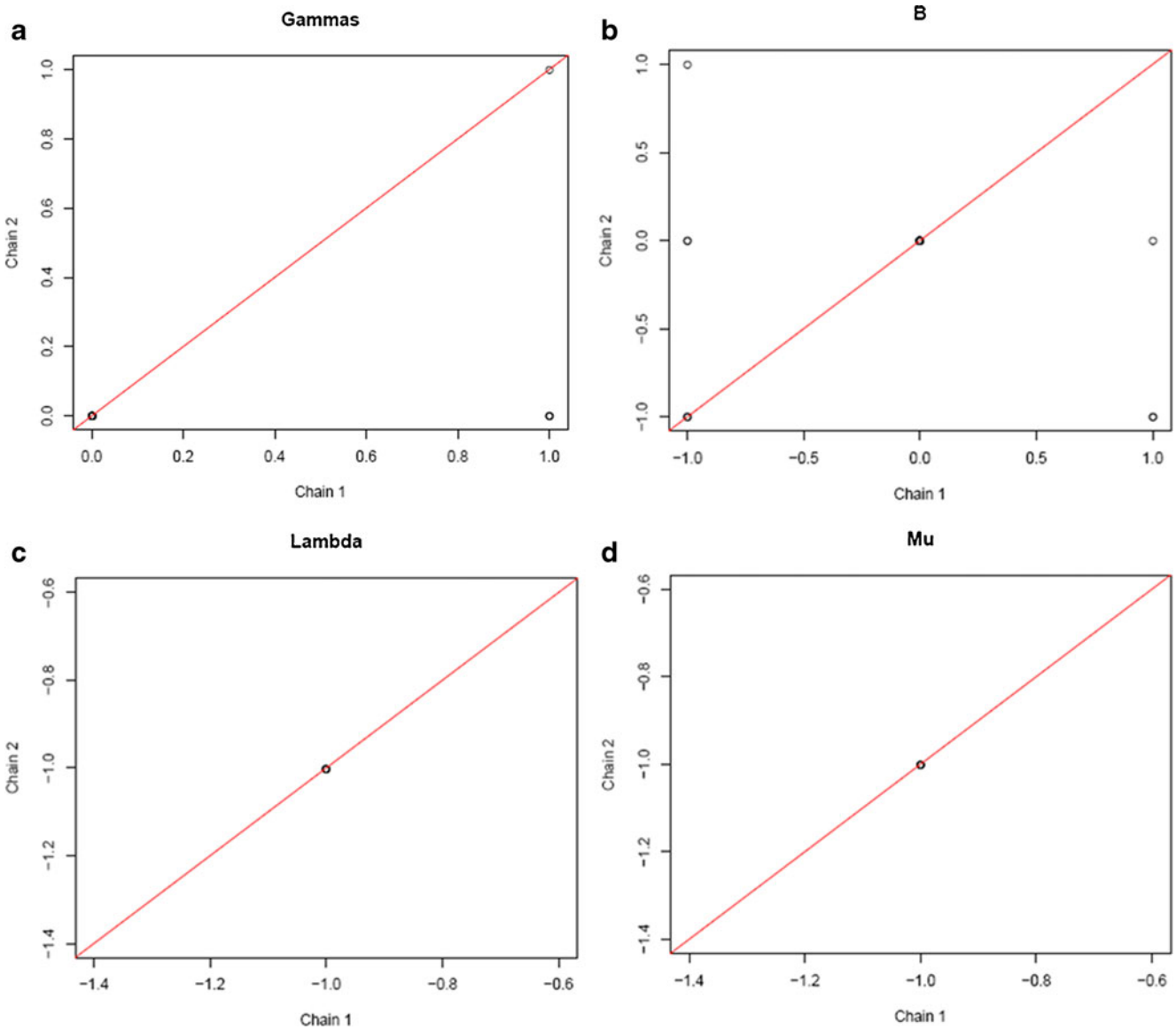


Fig. 8 **a** Indicator variable of Gibbs variable selection, **b** coefficient of linear regression, **c** precision of each regression, **d** intercept of each regression

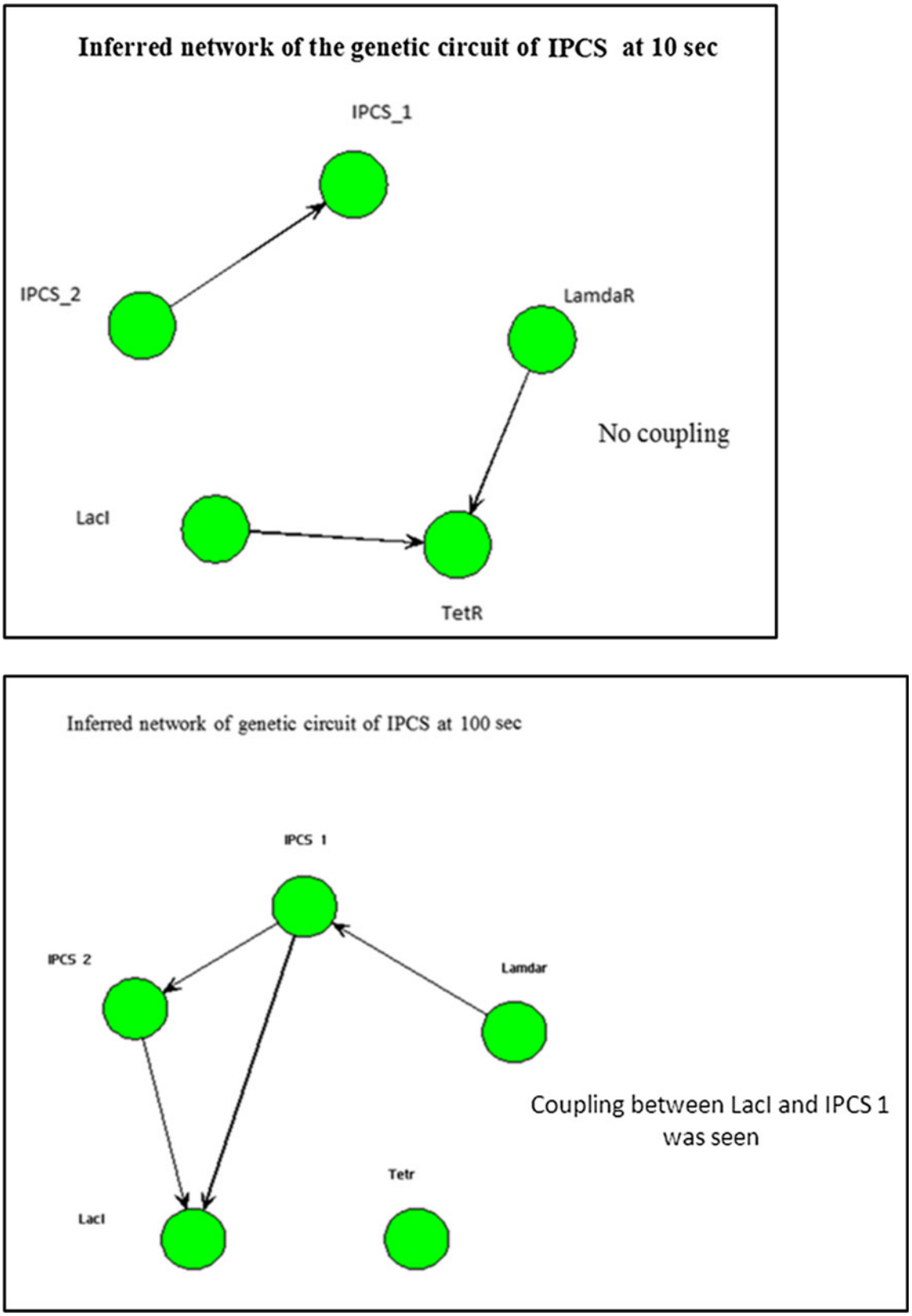


Fig. 9 Inferred network of the genetic circuit of IPCS showing the coupling between the genetic toggle switch and the repressilator

Table 5 Transition of states in the Boolean network for IPCS

Boolean network with five genes involved
Gene 1, gene 2, gene 3, gene 4, gene 5
Transition functions
Gene 1=<f (gene 3, gene 2, gene 4, gene 1, gene 5){11101101101111011111001111110000}>
Gene 2=<f (gene 3, gene 4, gene 5, gene 2, gene 1){00100001000010110100101000101011}>
Gene 3=<f (gene 5, gene 3, gene 1, gene 2, gene 4){10101110001010011000001111000110}>
Gene 4=<f (gene 4, gene 2, gene 3, gene 5, gene 1){11101010011101110011100010000111}>
Gene 5=<f (gene 3, gene 1, gene 5, gene 2, gene 4){00010011000101111110000001111011}>

Qualitative network modeling

To test the robustness of the circuit and identification of the number of attractors that circuit exhibits, qualitative Boolean network modeling for the genetic circuit was performed. After obtaining the steady state of the network, network was perturbed to check the robustness of the circuit. Boolean network for the circuit was constructed using time series data generated by COPASI. For the construction of Boolean network, binarization of the time series data was performed. The table containing the binarized time series data is included in the ESM.

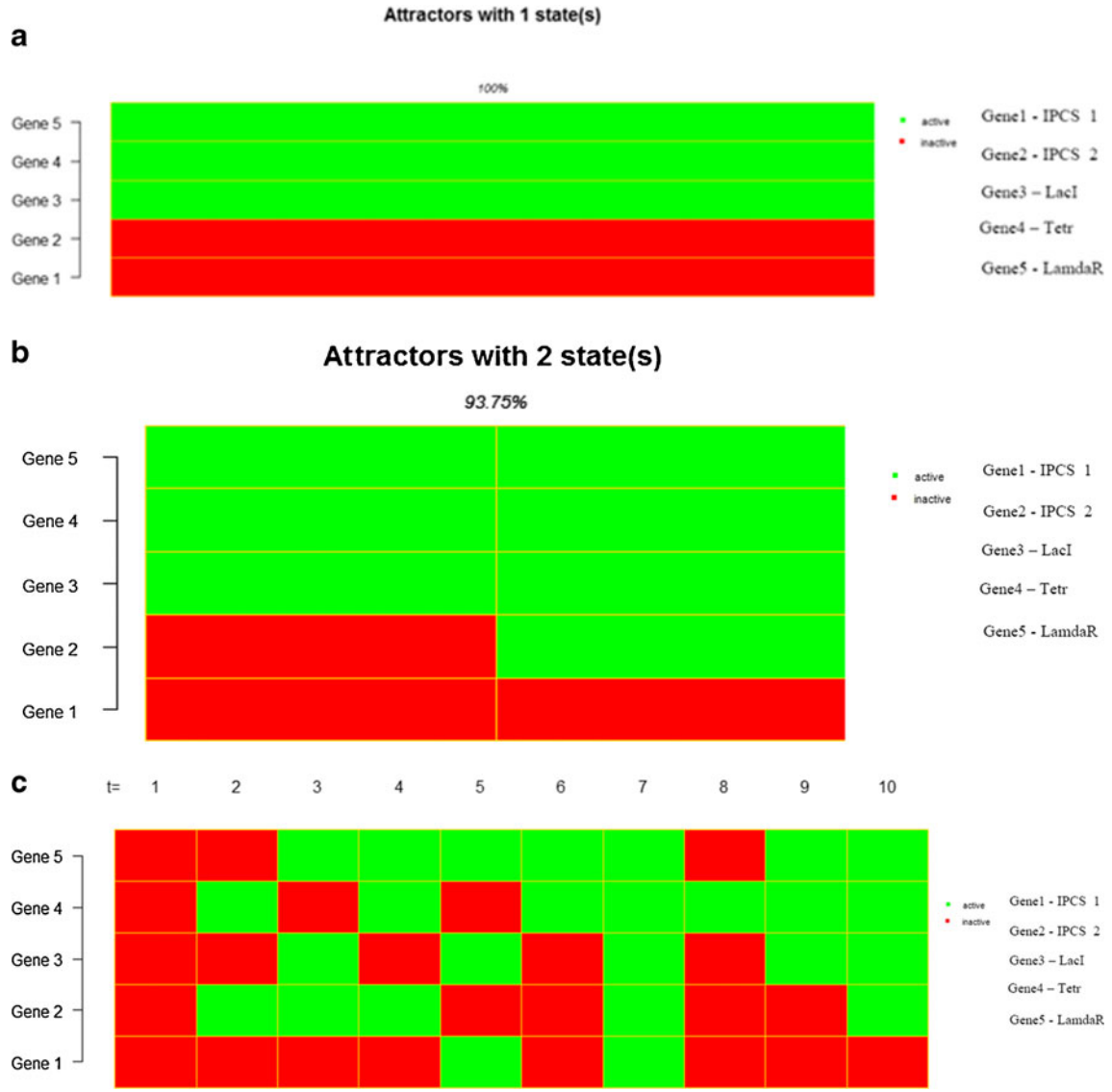


Fig. 10 **a** Attractor plot for the initial attractor of the network. **b** Attractor plot for two steady states. **c** Sequences of path to attractor

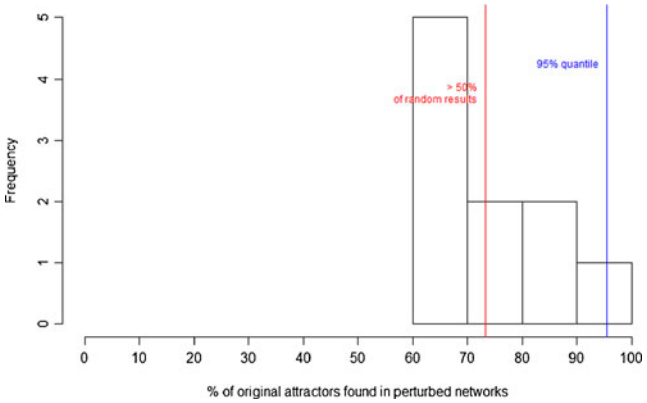


Fig. 11 Robustness plot of the genetic circuit

Boolean network

Boolean network was constructed from the time series data by using the best-fit algorithm. The number of states present in the Boolean network generated for IPCS was 32. These 32 states are represented by 1 (active state) and 0 (inactive state). State transition can be observed, after which the network circuit attains steady states (attractors). Genetic circuit had two attractors that indicate the bistability of the circuit constructed. The two steady states obtained represent the ON and OFF state for each gene in the circuit, 0 specifies the OFF state and 1 specifies the ON state. Attractor 2 is a simple attractor consisting of two state(s) and has a basin of 30 state(s). Genes are encoded in the following order: gene 1, gene 2, gene 3, gene 4, and gene 5. Transition of different states to obtain the steady state is represented by the transition table of the states which also includes the probability for each transition of the state (Table 5, ESM Table 1). The last state, 0 represents the OFF state for IPCS1 and ON state for IPCS2 and ON and OFF state for the other genes, respectively. This reveals the switching of genetic toggle switch of circuit where IPCS1 was repressed by IPCS2.

Attractors of the circuit are represented by the attractor plot which includes ON and OFF state for each gene in the network. In initial state, IPCS1 is ON, i.e., active state and IPCS2 is OFF, i.e., inactive state (Fig. 10a). LacI was in an OFF state, there was no coupling between LacI and IPCS1 resulting in repression of IPCS 2 by IPCS1. After the transition of the states, two steady states that were obtained are represented by an attractor plot which displays the two steady states justifying the dynamic behavior of the circuit to act as bistable genetic switch. The percentage to attain the steady states of the circuit was 93.75 % which reflects the robustness of the attractors (Fig. 10b). Attractor plot represents the OFF state of IPCS1 and ON state of IPCS2 which depicts the switching behavior of the circuit as IPCS2 represses IPCS1 which was not in observed in the initial state. This shows that the circuit toggles periodically between the two states and the two genes appear to repress each other mutually. This is in line with the hypothesis laid with the constructed genetic circuit for IPCS. Visualization of a sequence of states can be represented by the path of attractor's plot where the columns in the table represent consecutive states of the time series. The last state is the

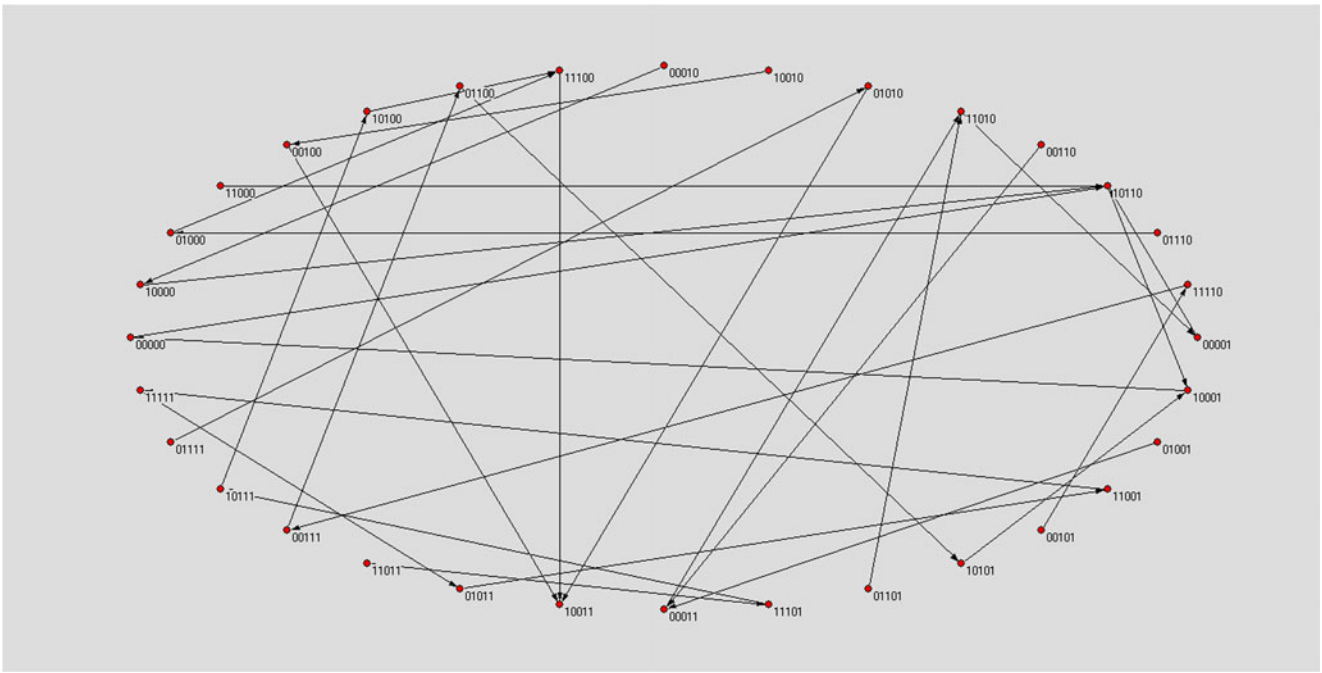


Fig. 12 State graph of the transition of states (visualized using Kamada–Kawai layout)

steady-state attractor of the network. This gives the inactive and active state of each gene at different time points (Fig. 10c).

Robustness of the genetic circuit

Perturbations in the genetic circuit are introduced to test the robustness of the networks to noise and mismeasurements. The robustness plot for the genetic circuit and the percentage obtained is greater than 50 % confirming the robustness of the constructed IPCS genetic circuit (Fig. 11). The network for the genetic circuit was exported to Pajek for the visualization of the transition states in the network (Fig. 12). Thus, the robustness plot of the circuit and the attractors obtained for the genetic circuit justifies the design of the IPCS circuit for *Leishmania*.

Conclusion

Genetic circuit designed for IPCS was subjected to simulation and validated by both qualitative and quantitative approaches. The circuit exhibited both bistable and oscillatory behavior along with robustness to perturbations. The methodology incorporated in this paper specifies those regions where the probability of observing the desired behavior is appreciable. This may allow a more comparative assessment of different design proposals for other protozoan parasites, especially when dynamics are expected or desired to exhibit elements of stochasticity. Collectively, the advances in the field of synthetic biology with the help of bioinformatics will help mitigate the great complexity and uncertainty currently impeding the study and design of synthetic circuits embedded in cellular systems. Such progress holds the promise of streamlining and intensifying the whole drug discovery process chain and aid delivery circuits as next generation therapeutics.

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